METHODS

Preparation of Mixed Keratinocyte and Melanocyte Cultures from Biopsy Specimens of Pigmented Skin Sites of Patients with Vitiligo

I. A. Usovetskii[1], V. V. Burunova[1], N. E. Kovtun[2,3], Yu. G. Suzdal'tseva[1], Yu. B. Krasil'nikova[1], N. G. Korotkii[1], and K. N. Yarygin[1,2,3]

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 148, No. 7, pp. 118-120, July, 2009
Original article submitted April 8, 2009

A method for preparation and expansion of mixed keratinocyte and melanocyte culture from human skin biopsy specimens was developed. The culture contains two melanocyte types: derivatives of hair follicle stem cells and epidermal basal layer stem cells. Fibroblasts were completely eliminated after culturing in selective medium.

Key Words: *melanocytes; keratinocytes; vitiligo*

Vitiligo is a disease of unknown etiology characterized by depigmentation of some skin sites caused by local absence of melanocytes (MC) in the epidermis. The number of patients suffering from this disease all over the world reaches several tens of millions. In many cases the disease leads to social disadaptation.

The known methods for the treatment of vitiligo are aimed at arrest or inhibition of the disease progress and pigmentation restoration in discolored skin site. According to recent data, the most effective method for pigmentation restoration is autotransplantation of a mixture of keratinocytes (KC) and MC [5-7,10,11,13] or MC alone [1-3,9,10]. This approach is effective in 65-100% cases with focal and segmented vitiligo, which is much better than the efficiency of other methods, both conservative and surgical. Even in generalized form pigmentation is restored in 15-30% patients. Unfortunately, in Russia transplantations of MC suspensions are not practiced in vitiligo patients up to the present time.

According to published data, two variants of the KC and MC mixture are used in therapy: fresh suspension from biopsy specimens of pigmented skin sites or suspension obtained after co-culturing of these two cell types [5-7,10,11,13]. Purified MC suspension (without KC) is prepared from MC monoculture [1-3,9,10]. The advantage of the approach including the culturing stage is that due to *in vitro* expansion of cells, the area of the skin flap used for preparation of the transplant is significantly lower.

We obtained mixed KC and MC culture from pigmented skin sites of vitiligo patients, characterized and optimized the conditions of MC expansion. The aim of our next study will be to evaluate the efficiency of the prepared cultures transplantation to patients with segmented vitiligo.

[1]Russian State Medical University; [2]V. N. Orekhovich Institute of Biomedical Chemistry, Russian Academy of Medical Sciences; [3]Institute of General Pathology and Pathophysiology, Russian Academy of Medical Sciences, Moscow, Russia. ***Address for correspondence:*** burunova@mail.ru. V. V. Burunova

0007-4888/09/1481-0103 © 2009 Springer Science+Business Media, Inc.

MATERIALS AND METHODS

Biopsy specimens were collected with a perforator No. 4 (Miltex) from the gluteal area of patients with vitiligo. Before biopsy, the gluteal skin was exposed to UV light 3-4 times at 2-3-day intervals in one erythema dose needed for the appearance of slight, but clearly outlined erythema. This biological dose was selected individually for each patient using the Gorbachev—Dolfeld biodosimeter. Exposure of biopsy zones was carried out before the development of stable sunburn.

The method for obtaining mixed KC and MC culture is based on the classical procedure [14], with significant modifications. Biopsy specimens were washed several times in Hanks' solution (PanEco) with antibiotics and antimycotic (Gibco), after which the material was cut with sterile ophthalmic scissors into small fragments (2-4 mm), treated in a mixture of 0.1% dispase and 0.1% collagenase-1 (Gibco) for 18 h at 4°C. Skin fragments were separated into the derma and epidermis with fine pincers. The epidermis was additionally treated with 0.25% trypsin (PanEco) for 20 min at 37°C. After inhibition of trypsin with fetal calf serum (Gibco), the processed epidermis was filtered through sterile Nylon filter. The filtrate was centrifuged at 300*g* for 5 min, the precipitate was resuspended in complete growth medium for KC (Defined K-SFM) with 10% fetal calf serum (Gibco), and the resultant cell suspension was inoculated (200-300 thousand cells/ml) in collagen-coated culture flasks (Greiner). Cell concentration was determined in a Goryaev's chamber, cell viability was evaluated by trypan blue exclusion. The suspension contained, besides the cells, hairs with or without follicles.

RESULTS

Foci of KC growth appeared as early as on day 1 of culturing (Fig. 1, *a*). These foci initially consisted of 10-40 cells, later they grew in size. The culture also contained solitary KC scattered over the entire surface of the culture flask (Fig. 1, *b*). On days 3-5, MC could

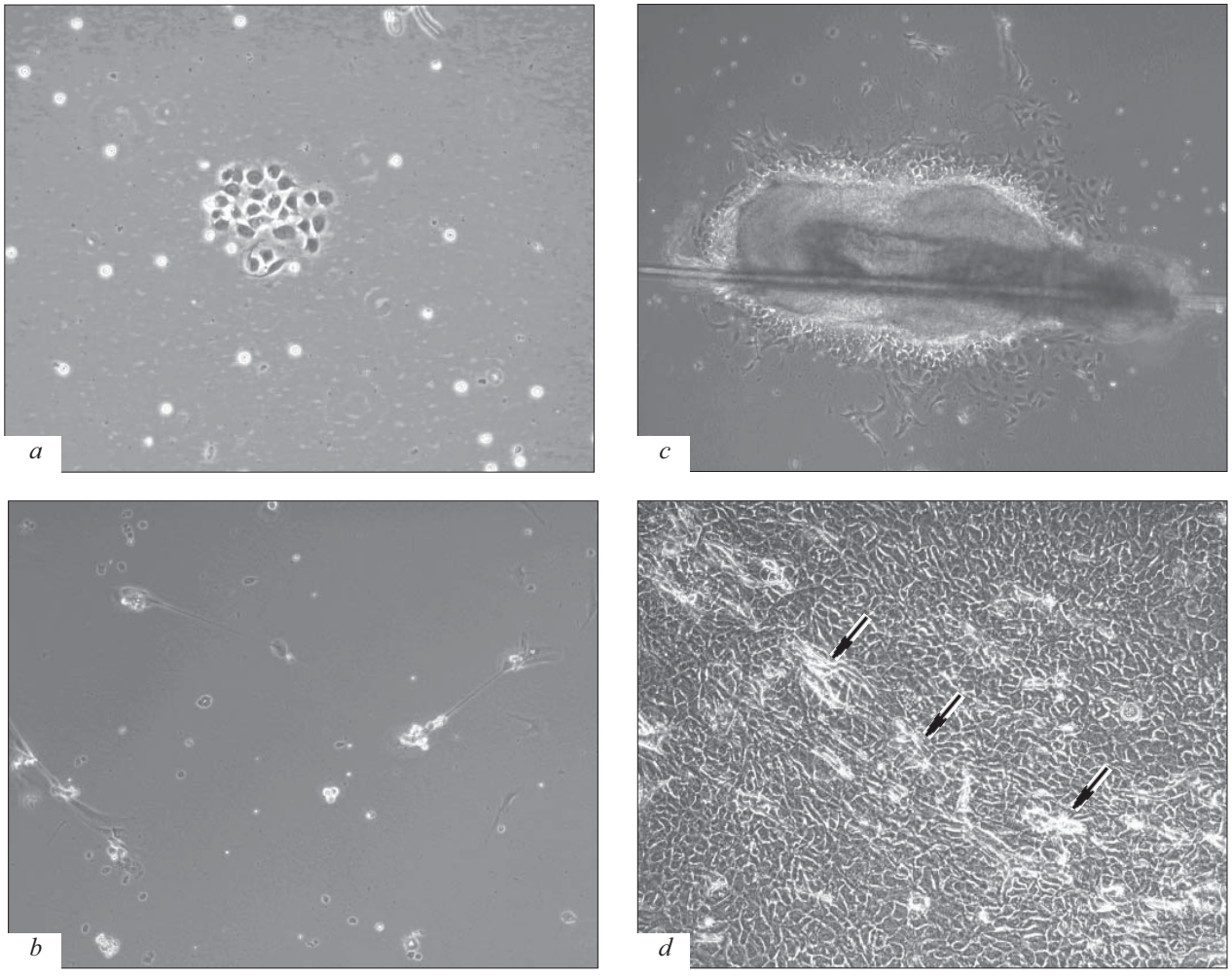

Fig. 1. Mixed KC and MC culture from human skin. a) day 1 of culturing, foci of KC growth; b) day 3 of culturing, MC; c) day 5 of culturing, MC and KC are released from a hair follicle; d) day 14 of culturing. Monolayer. Arrow shows an MC "crawling" over KC.

be identified in the culture. Some MC were solitary or formed groups of 2-4 cells (Fig. 1, *b*), others concentrated around hair follicles together with KC (Fig. 1, *c*). Melanocytes could be easily identified by characteristic morphology.

The culture contained fibroblasts which were then eliminated, while KC and MC were evenly distributed over the surface; MC tended to "crawl" over KC, particularly in subconfluent and confluent cultures (Fig. 1, *d*). Fresh culture formed a complete monolayer after 2-3 weeks. By this time, the content of MC was 4-7% of the total cell count.

A total of $10\text{-}50\times10^6$ cells with viability of at least 96% were obtained from the biopsy specimen collected as described above. The culture could be subjected to expansion by passages.

Hence, we obtained fibroblast-free mixed KC and MC culture with a high content of MC.

According to published data, human skin contains two MC types with precursor cells located in the epidermal basal layer (epidermal MC, EMC) and in hair follicles (hair follicle MC, HFMC) [12]. According to the classical concepts, EMC precursors normally serve as the source of skin repigmentation, while HFMC progenitors represent a reserve pool of MC, which start active multiplication and differentiation only under the effect of intensive UV exposure [4,12,15]. Presumably, HFMC play the key role in skin pigmentation in response to UV exposure and in its repigmentation in vitiligo [12,15]. KC precursors are also located in the epidermal basal layer and in the hair follicles; the former serve as the source of cell material for regeneration of the epidermis in health, while the latter are involved in restoration of damaged epidermis.

It is known that all intact somatic cells are characterized by a limited multiplication potential, limited by the so-called Hayhflick limit [8], because of progressive shortening of telomeres in successive divisions and absence of telomerase activity in somatic cells (telomerase is an enzyme restoring the telomeres). It is shown that shortening of telomeres in HFMC is slower than in EMC. Hence, the cultures obtained in our studies contain EMC (Fig. 1, *b*) and HFMC (Fig. 1, *c*), and it is highly possible that they will be effective in the treatment of vitiligo. It is noteworthy that some HFMC are eliminated in MC culturing without KC by some methods, which can explain the differences in the efficiency of MC culture transplantation, according to different authors.

Our cultures contain two types of KC and their progenitors: from the epidermal basal layer and from hair follicles. Before transplantation of KC and MC mixture to vitiligo patients depigmented skin sites are usually pretreated (the epidermis is removed). The presence of KC and their precursors from hairy follicles in mixed culture is expected to provide rapid physiological recovery of the epidermis.

REFERENCES

1. R. Czajkowski, *Dermatol. Surg.*, **30**, No. 11, 1400-1405 (2004).
2. R. Czajkowski, W. Placek, T. Drewa, *et al.*, *Ibid.*, **33**, No. 9, 1027-1036 (2007).
3. Y. F. Chen, P. Y. Yang, D. N. Hu, *et al.*, *J. Am. Acad. Dermatol.*, **51**, No. 1, 68-74 (2004).
4. J. Cui, L. Y. Shen, and G. C. Wang, *J. Invest. Dermatol.*, **97**, No. 3, 410-416 (1991).
5. R. Falabella, C. Escobar, and I. Borrero, *J. Am. Acad. Dermatol.*, **26**, No. 2, Pt. 1, 230-236 (1992).
6. Y. Gauthier and J. E. Surleve-Bazeille, *Ibid.*, 191-194.
7. S. Gupta, A. Goel, A. J. Kanwar, and B. Kumar, *Int. J. Dermatol.*, **45**, No. 6, 747-750 (2006).
8. L. Hayflick and P. S. Moorehead, *Exp. Cell Res.*, **25**, 585-591 (1961).
9. S. Im, S. K. Hann, Y. K. Park, and H. I. Kim, *Yonsei Med. J.*, **33**, No. 4, 344-350 (1992).
10. W. Lontz, M. J. Olsson, G. Moellmann, and A. B. Lerner, *J. Am. Acad. Dermatol.*, **30**, No. 4, 591-597 (1994).
11. S. V. Mulekar, *Int. J. Dermatol.*, **44**, No. 10, 841-845 (2005).
12. G. Y. Na, S. H. Park, B. C. Park, *et al.*, *Br. J. Dermatol.*, **155**, No. 5, 902-909 (2006).
13. E. Pianigiani, M. Risulo, A. Andreassi, *et al.*, *Dermatol. Surg.*, **31**, No. 2, 155-159 (2005).
14. J. G. Rheinwald, *Methods Cell. Biol.*, **21A**, 229-254 (1980).
15. R. G. Staricco, *Ann. N. Y. Acad. Sci.*, **100**, 239-255 (1963).